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On the role of ephrinA2 in auditory function

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Abstract

Recent findings suggest that the manipulation of the EphA/ephrinA system can improve hearing threshold sensitivity in the auditory system (Yates et al., 2014). These results appear to open-up the possibility that pharmacological manipulation of this system could lead to the development of treatments to cure some types of hearing loss. As a first step towards this goal, we have performed a further series of auditory brainstem evoked potential recordings on ephrinA2 homozygous knockout mice and their wildtype littermates in order to replicate the previously reported findings. However, we found that ephrinA2 knockout mice had auditory threshold sensitivity for click and 3-42 kHz tone pip frequencies comparable to that of their wildtype littermates. Evoked potential wave amplitudes, latencies and inter-peak intervals were also comparable between ephrinA2 knockout mice and wild type control littermates. Thus in our experiments we could not replicate the findings of Yates et al. (2014). Whilst the EphA/ephrinA system may therefore play a role in the development of innervation of the cochlea and neural circuitry of the auditory brainstem, there appears to be a functional redundancy between members of this family such that loss of ephrinA2 function alone is insufficient to alter auditory function in the mouse.

Highlights

- published findings of improved hearing in ephrinA2 knockout could not be replicated
- ephrinA2 knockout mice had comparable auditory sensitivity to littermate controls
- evoked potential wave amplitudes were normal in ephrinA2 knockout mice
- wave latencies and interpeak intervals were normal in ephrinA2 knockout mice

Keywords

Auditory Brainstem Response, Evoked Potentials, Mouse, EphA/ephrinA, Hearing Thresholds, Auditory System.

Abbreviations (Define non-standard abbreviations)

ABR : Auditory Brainstem Response

Introduction

Despite the major impact of hearing loss in the human population (Davis, 1995), there is relatively little known about the molecular basis underlying the development and maintenance of hair cell innervation in the cochlea, and therefore it is difficult to devise treatment strategies to reverse the loss of this innervation or to stimulate new axon/dendrite growth. Candidate molecules to contribute to these processes include members of the EphA family of receptor tyrosine kinases and their ephrinA ligands (Kania and Klein, 2016), which have been shown to influence the innervation pattern of both inner and outer hair cells in the cochlea (Defourny et al., 2013). EphrinA2 in particular has been shown to have prominent expression in many structures surrounding the cochlear duct and in the spiral ganglion of the developing mouse cochlear (Pickles et al., 2002). Recently, it has been reported that mice with a knockout of ephrinA2 show improved auditory thresholds at high stimulus frequencies compared with control mice, and with double ephrinA2/A5 knockouts showing even better thresholds (Yates et al., 2014). This is the first time that a single gene disruption has been reported to lead to better auditory function, while mutations of over 300 other genes lead to worse hearing.

Members of the Eph family, the largest family of receptor tyrosine kinases, are predominantly expressed in the developing and adult brain, and are involved in almost all processes of neural circuit development of sensory systems, from the initial establishment of neuronal connectivity to the control of synapse function (Kania and Klein, 2016). In the retino-collicular projection, for example, multiple ephrinAs and EphAs are expressed in both the retina and in the superior colliculus (SC), and are crucially involved in establishing a topographic projection which enables the transfer of positional information between these regions (Suetterlin et al., 2012; Triplett and Feldheim, 2012). These EphA/ephrinA modules are found also in other centres transferring and processing visual information such as the lateral geniculate nucleus (LGN) and the visual cortex, and guide the establishment of the retino-geniculate-, thalamo-cortical- and cortico-collicular projection. Likewise, EphA/ephrinA modules have been identified in the auditory system in nuclei involved in sound processing including the auditory cortex, the medial geniculate nucleus (MGN) and the olivary nucleus. However, their functional characterisation is here less advanced (Torii et al., 2013).

There are many different causes of hearing impairment, including genetic variants and environmental traumas such as noise or drug-induced damage. Various parts of the auditory system can be affected, but recently particular attention has focussed on the innervation of the primary

sensory receptor cells (inner hair cells, IHC) of the cochlea. Each IHC is innervated by around 10-20 unbranched dendrites of type I cochlear afferent neurons with cell bodies in the spiral ganglion of the cochlea. These are bipolar neurons, with their axons forming the cochlear nerve. Other types of afferent neurons (type II) branch and innervate the outer hair cells (OHCs), and efferent neurons also form synapses with OHCs or with IHC afferent neurons. Among the neurons innervating a single IHC, some have a high spontaneous firing rate and low threshold while others have low spontaneous rates and high thresholds (Liberman, 1982). Preferential damage to the latter subset of IHC afferent dendrites due to noise exposure or ageing can lead to "hidden hearing loss", where auditory sensitivity (thresholds for detecting sounds) is unaffected but the loss of high threshold innervation leads to reduced signalling amplitudes and difficulties in processing supra-threshold features (Kujawa and Liberman, 2009; Sergeyenko et al., 2013). This is thought to be associated with the difficulty people find in following speech in noisy backgrounds, and may also lead to other auditory pathologies such as tinnitus. The damage to IHC afferent dendrites in the animal models used appears to be irreversible and ultimately leads to degeneration of the neuron.

To date, almost all mouse mutants studied to understand the workings of the auditory system and the molecular underpinnings of deafness show worse thresholds for sound detection. From the unexpected report of improved hearing sensitivity in an ephrinA2 mouse knockout (Yates et al., 2014), we hypothesised that this effect was due to cochlear nerve mis-wiring. The novel finding that hearing sensitivity was improved after the loss of function of a particular protein made a therapeutic intervention much more promising, to slow down or reverse the progression of hearing impairment under certain circumstances.

Here, we aimed to confirm the published findings (Yates et al., 2014) that loss of functional ephrinA2 protein in the mouse leads to improved hearing thresholds, measured electro-physiologically. However, we found no difference in any feature of the auditory brainstem response (ABR) between mutants and their littermate controls.

Materials and methods

These studies were carried out in accordance with UK Home Office regulations, the UK Animals (Scientific Procedures) Act of 1986 (ASPA) under UK Home Office licences, and EU Directive 2010/63/EU for animal experiments. The work was approved by King's College London Ethical Review Committees. At the completion of electrophysiological recordings, mice were culled using methods approved under these licences to minimize any possibility of suffering.

We examined mice with a knockout of ephrinA2 on a C57BL/6 genetic background (Feldheim et al., 2000). This was the same allele (*Efna2*^{tm1Jgf}) as used by the earlier report (Yates et al., 2014). Mice were bred and housed within a specific pathogen free animal unit at King's College London. For this study, littermates from a cross between ephrinA2 heterozygous knockout mice were analysed. These mice were genotyped according to protocols described in Feldheim et al. (2000).

For electrophysiological testing, we recorded auditory brainstem responses (ABR) following the protocol of Ingham et al (2011). Mice were anaesthetised with a 10 ml/kg ip injection of a Ketamine/Xylazine mixture (containing 10 mg ketamine / 10 g bodyweight and 0.1 mg Xylazine / 10 g bodyweight) and placed into a warm cage. Once anaesthetised, the mouse was placed onto a heating blanket (Harvard Apparatus) inside a sound attenuating chamber (IAC Ltd, Mini-Acoustic Chamber MAC1). Needle electrodes were placed subcutaneously around the mouse's head; a ground electrode over the right bulla, a reference electrode over the left bulla and an active electrode on the vertex. The mouse was then placed in a natural prone position facing a loudspeaker, at a distance of 20 cm from the leading edge of the speaker to the animal's interaural axis.

A custom software application, driving Tucker Davis Technologies (TDT) System 3 hardware (RP2.1, RA16, PA5) was used for acoustic calibration, presentation of stimuli and recording of evoked potential responses. Stimuli were calibrated using a PCB Piezotronics Inc. microphone system (Model 378C01 condenser microphone, Model 426B03 preamplifier and Model 480C02 signal conditioner) and presented in dB SPL re. 20 µPa. Stimuli consisted of tone pips, 5 ms duration, at frequencies of 3, 6, 12, 18, 24, 30, 36 & 42 kHz with a 1 ms rise/fall time and clicks (10 µs duration). These were generated in software at a sample rate of 97.656 kHz, converted to the analogue domain (TDT RP2.1), attenuated to achieve the desired final sound level (TDT PA5), amplified (TDT SA1) and presented as groups of 256 stimuli at 42.6/sec at 5 dB increments from 0 to 95 dB SPL, via a CTS

Type 241 transducer (RS components). Evoked potentials detected by the subcutaneous electrodes in response to the stimuli were amplified and digitised (TDT RA4LI headstage and RA4PA preamplifier) before being digitally filtered (TDT RA16; 300-3000 Hz) further amplified and stored in an averaging buffer at a sample rate of 97.656 kHz. The auditory brainstem response (ABR) was saved in software as a 20 ms duration averaged response to 256 presentations of each acoustic stimulus.

ABR thresholds for each click and tone stimulus were estimated from the data recorded as the lowest sound level that evoked a recognisable portion of the overall ABR waveform by visual inspection of responses stacked according to sound level (see Figs. 1A,B for examples). ABRs were examined in detail, extracting positive and negative peak amplitudes and latencies, using software routines kindly provided by M.C. Liberman (ABR Notebook). From these values, the peak-peak amplitudes of the first 4 waves of each ABR (P1-N1, P2-N2, P3-N3, P4-N4; see Figs. 1A,B, Figs. 2A,B,C) were calculated along with the interpeak intervals from positive wave 1 to subsequent positive peaks (P1-P2, P1-P3 & P1-P4 intervals).

Results

ABR recordings were made from 15 wildtype mice of both genders, with a mean age of 55.7±4.2 days (21.7 ±2.6 g bodyweight) and from 24 littermate ephrinA2 knockout mice of both genders with a mean age of 55.6±4.2 days (20.6 ±5.2 g bodyweight). All recordings and analyses were performed by a single experimenter (NI) to ensure good reproducibility between mice and recordings were performed over 3 experimental days to reduce variability in laboratory conditions over longer periods.

ABR thresholds measured from wildtype and KO mice, for all stimuli tested, were found to be comparable (Fig. 1C). There was no statistically significant effect of genotype on thresholds measured in the 2 populations (Mann-Whitney rank sum test, T=24755.0, p=0.28).

We examined the ABR waveforms from wildtype and KO mice in detail (Fig. 2). Mean ABR waveforms, averaged across all mice of each genotype, recorded at 40 dB SL (sensation level; i.e. dB above threshold), were exceptionally similar for all stimuli tested. Figs. 2A-C illustrates this observation using click, 12 kHz and 24 kHz stimuli respectively. Responses from 6 kHz, 18 kHz and 30 kHz stimuli are not shown, but were equally similar across genotype.

We quantified the positive and negative peak amplitudes and latencies from individual mice for each stimulus and sound level relative to threshold. Mean peak-peak amplitudes for the first 4 clearly distinguished peaks as a function of dB SL are plotted in Fig. 2D-F, for click, 12 kHz and 24 kHz stimuli respectively. These features were comparable in wildtype and KO mice; amplitude increased with the same pattern of growth in both genotypes for the different stimuli tested. Response amplitudes evoked by 6 kHz, 18 kHz and 30 kHz stimuli are not shown, but were equally similar across genotype.

In a similar way, there were no genotype-related differences of the absolute latency of the positive and negative peaks of the 4 waves (P1, N1 to P4, N4), plotted as mean latency as a function of stimulus level (Fig.2 G-I), nor of the interpeak intervals between P1 and subsequent peaks (P1-P2, P1-P3, P1-P4) as a function of stimulus level (Fig. 2J-L), for any stimulus tested.

In Fig. 3, measurements taken from 40 dB SL responses were plotted as a function of stimulus (click, or tone-pip frequency). Mean amplitudes of waves 1-4 are plotted in Fig. 3A-D. The mean click evoked amplitude is larger for each wave than for tone-evoked amplitudes. This is consistent with

the fact that a broadband click evokes afferent activity across a wider range of auditory nerve fibres, producing a larger amplitude response. However, there was no statistically significant effect of genotype for any wave amplitude evoked by any stimulus measured from the 2 populations (t-test; $p>0.05$ in every comparison). Fig. 3E plots the mean latency of the peak of wave 1 for clicks and tone-pip stimuli. The mean click-evoked P1 latency is reduced compared to tone-evoked responses and is consistent with broadband stimulation of the cochlea. It is also evident that mean P1 latency is smaller for higher frequency tones (e.g. 30 kHz) compared to lower frequency tones (e.g. 6 kHz). This difference (approximately 300 µs) is consistent with the differences in cochlear delay of the traveling wave in stimulation of apical versus basal regions of the cochlea. Mean inter-peak intervals are plotted in the same way in Fig. 3F-H. There was no statistically significant effect of genotype for any comparison of P1 latency or for comparison of the inter-peak intervals from the 2 populations (t-test; $p>0.05$ in every comparison).

Further to the data shown in Fig. 3, we pooled values from individual mice across each of the tone-pip frequencies measured for 3 parameters: amplitude, P1 latency and inter-peak interval. A comparison of these pooled data using t-tests showed that there was no statistically significant effect of genotype for any parameter.

Discussion

Numerous investigations have highlighted the prominent role of the ephrinA system in the development of sensory modalities including the visual-, the olfactory-, vomeronasal-, the somatosensory- and taste systems but also the auditory system (Cramer et al., 2000), suggesting that the Eph family overall might have a principal role in the patterning of possibly all early sensory axonal projections.

Intriguingly, recent functional data showed that a knockout of ephrinA2 apparently did not lead to the typically observed disruption of circuit function (e.g. Feldheim et al., 2000) but in contrast to an increase in hearing sensitivity suggesting an improvement in functions of the auditory neural circuit (Yates et al., 2014). This tempted us to explore in further detail the role of this ligand in the auditory system. Here we show, however, that ephrinA2 knockout mice aged 8 weeks old do in fact have a normal auditory threshold sensitivity and normal neural brainstem response patterns when compared to age-matched wildtype littermate control mice.

The study by Yates et al. (2014) showed significant threshold improvements of approximately 17 dB for ephrinA2 knockout mice compared to controls, for both 24 kHz and 30 kHz stimuli. In a ephrinA2/A5 double knockout, even greater threshold improvements of approximately 24 dB at 24 kHz, and 27 dB at 30 kHz were found. A knockout of ephrinA5 only alone did not produce any alterations in threshold sensitivity (Yates et al., 2014). Furthermore, a range of significant effects of the single or double knockouts with regard to the evoked potential amplitudes and latencies were reported. However, our own investigation of ephrinA2 knockout mice do not support the results of Yates et al., (2014) as we found near-identical response features of ephrinA2 knockout mice and their wildtype littermate controls.

Several factors may contribute to the differences in the results seen for the ephrinA2 knockout mice.

(1) There are some differences in how the ABR were recorded in the two studies. Yates et al. (2014) presented stimuli through a coupler to the opening of the left ear canal. As such, the responses recorded are likely to be predominantly monaural in nature. In our study, we used free-field presentation, resulting in binaural stimulation. In both cases, the evoked potentials were recorded between the vertex and the left mastoid/bulla. However, while being a possibility, there is no *a priori* reason to suggest that these methodological differences could account for the differences seen in the two investigations.

(2) The ephrinA2 knockout mouse line used in the current study here carries the same allele as that used by Yates et al. (2014) (Feldheim et al., 2000). Thus, the apparent differences in hearing sensitivities cannot be explained on the basis of using two different ephrinA2 mutations. A subtle effect of genetic background due to genetic drift, however, cannot be discounted as a source of the discrepancy in the findings between their and our studies.

(3) Yates et al. (2014) did not use wildtype littermate controls. Despite the fact that their knockout mice were on the same C57BL/6J genetic background as ours, and had been backcrossed onto this background for 15 generations, it remains a limitation of their experimental design that littermate wildtype controls were not used.

(4) The data reported in the current study were collected from young age-matched mice (ephrinA2 knockout, 55.6 ±4.2 days, and wildtype control mice, 55.7 ±4.2 days, with age ranging from 50 to 60 days across all mice tested). This age was chosen to avoid any complications from the age-related progressive high-frequency hearing loss, which starts to become apparent from between 3-6 months old mice partly due to the presence of the *Cdh23*^{ahl} allele in C57BL/6 mice (Kane et al., 2012). Yates et al. (2014) did not use wildtype littermate controls and tested mice over a much wider span of age: 14.2 ±4.8 weeks for their wildtype population, 22.6 ±11.0 weeks for the ephrinA2/5 double knockout mice, and 19.9 ±11.6 weeks for the ephrinA2 mutant mice. Thus comparing auditory data from C57BL/6J mice across a range of ages is another limitation of their experimental design. We have focussed on the developmental role played by ephrinA function in establishing correct wiring patterns and circuitry in the brain and chose to work on young mature mice (8 weeks old). However, it may be that ephrinAs do play a role also in maintaining features such as auditory sensitivity and tonotopicity in the auditory system in advanced ages, and as such we may not see such effect in our young knockout mice.

Overall, it appears most likely that the differences in the data reported by us compared to those of Yates et al. (2014) are attributable to differences in the age ranges of the mice tested, and - to a lesser extent - due to a lack of littermate wildtype controls which will lead to variations due to different environmental conditions as well as age to influence the results. The apparent lack of phenotype in the auditory brainstem response in ephrinA2 knockout mice in our study might indicate a functional redundancy between the members of the ephrin family or other molecules

involved in early neural circuit formation, similar to findings made in the retino-collicular projection (Suetterlin et al., 2012), the most likely candidate here being ephrinA5.

The finding that the knockout of a single gene (or the knockout of a pair of related genes) could lead to an improvement in hearing threshold sensitivities came as a surprise. As of today, there are no other examples of such a finding. In fact, all published data report the opposite effect where genetic mutations lead to a worse hearing sensitivity. This argues that a knockout producing an improvement in hearing thresholds would be an exceptionally rare case. The search for genes responsible for improved hearing in mammals must therefore continue.

Conclusions

We were unable to reproduce the findings by Yates et al. (2014), who reported that a knockout of ephrinA2 leads to an improvement in hearing threshold sensitivity. Our results presented here show that in the young mature mouse, all parameters associated with measurement of the auditory brainstem response (threshold sensitivity, waveform shape, wave amplitude, wave latency and inter-peak interval) are not statistically significant different between ephrinA2 knockout mouse and littermate wildtype controls. The concept that pharmacological manipulation of biochemical pathways (signalling, metabolic, neural, etc.) could one day help to reverse hearing impairment is nevertheless intriguing and worthy of continued further investigation.

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Figure legends

Fig. 1. Auditory brainstem responses and thresholds in EphrinA2 knockout mice. Auditory Brainstem Response recordings from (A) wildtype and (B) EphrinA2 knockout mice obtained in response to 12 kHz tone pips presented from 0 – 95 dB SPL in 5 dB increments. Five positive peaks of the waveforms are labelled P1 – P5. However, wave 5 was more variable and less consistent across stimulus level compared to waves 1 – 4. Therefore, detailed analyses of wave 5 were not performed due to the less reliable appearance of this peak The visually-determined threshold for these responses is indicated by the emboldened line. (C) Mean (± standard deviation) thresholds for ABRs evoked by clicks and 3-42 kHz tone pips are shown for wildtype mice (black symbols; n=15) and for EphrinA2 knockout mice (red symbols; n=24). A Mann-Whitney rank sum test suggested there was no statistically significant effect of genotype on the thresholds estimated from the 2 populations (T=24755.0, p=0.28)

Fig. 2. Auditory brainstem response waveform shapes and changes in amplitude, latency and interpeak intervals with stimulus level. In each panel, responses from wildtype mice are indicated by black lines & symbols; responses from EphrinA2 knockout mice are indicated by red lines & symbols. Panels A-C indicate averaged ABR waveforms for stimuli at 40 dB SL (sensation level), pooled across all wildtype mice and all EphrinA2 KO mice, for clicks (A), 12 kHz (B) and 24 kHz (C). The four positive and negative peaks used for later analyses are indicated by P1-N1 to P4-N4. Panels D-F plot the growth of waves 1-4 as a function of suprathreshold stimulus level (dB SL) for clicks (D), 12 kHz (E) and 24 kHz (F). Amplitudes (μV) are plotted as mean (± standard deviation). Panels G-I plot the change in latency of the 4 positive and negative peaks as a function of suprathreshold stimulus level (dB SL) for clicks (G), 12 kHz (H) and 24 kHz (I). Latencies (ms) are plotted as mean (± standard deviation). Panels J-L plot the change in interpeak interval between P1 and P2-P4 as a function of suprathreshold stimulus level (dB SL) for clicks (J), 12 kHz (K) and 24 kHz (L). Intervals (ms) are plotted as mean (± standard deviation). No obvious differences were noted between the wildtype and knockout mice for any of these parameters. ABRs evoked by 6 kHz, 18 kHz and 30 kHz tone pips were analysed in the same way but are not illustrated here.

Fig. 3. Auditory brainstem response amplitude, latency and interpeak interval as a function of stimulus frequency at 40 dB SL. All parameters plotted here were recorded at 40 dB above threshold. In each panel, responses from wildtype mice are indicated by black lines & symbols; responses from EphrinA2 knockout mice are indicated by red lines & symbols. Panels A-D plot mean peak-peak amplitude (± standard deviation), for click and 6-30 kHz tone pip stimuli, for wave 1 (P1-

N1; A), wave 2 (P2-N2; B), wave 3 (P3-N3, C) and wave 4 (P4-N4, D). For each stimulus and wave, amplitudes recorded in wildtype and EphrinA2 knockout mice were compared using a t-test. There was no statistically significant effect of genotype for any of the wave amplitudes evoked by any stimulus measured from the 2 populations. Panels E-H plot the mean time (± standard deviation), for click and 6-30 kHz tone pip stimuli, for the absolute latency of P1 (E), and the interpeak intervals of P1–P2 (F), P1-P3 (G) and P1-P4 (H). For each stimulus and interval, measurements taken from wildtype and EphrinA2 knockout mice were compared using a t-test. There was no statistically significant effect of genotype for any of the wave latencies or intervals evoked by any stimulus measured from the 2 populations. Results of two-tailed t-tests for each parameter were as follows (t-Statistic & corresponding p-values for 37 degrees of freedom): (A) P1-N1 Amplitude; Click, t=-0.139, p=0.890; 6kHz, t=-0.731, p=0.470; 12kHz, t=-0.037, p=0.971; 18kHz, t=-0.294, p=0.770; 24kHz, t=0.230, p=0.819; 30kHz, t=1.180, p=0.247. (B) P2-N2 Amplitude; Click, t=0.171, p=0.865; 6kHz, t=-1.192, p=0.241; 12kHz, t=-1.265, p=0.214; 18kHz, t=-0.182, p=0.856; 24kHz, t=-0.285, p=0.777; 30kHz, t=-0.082, p=0.935. (C) P3-N3 Amplitude; Click, t=0.329, p=0.744; 6kHz, t=0.409, p=0.685; 12kHz, t=-0.323, p=0.745; 18kHz, t=0.117, p=0.907; 24kHz, t=-1.193, p=0.240; 30kHz, t=0.529, p=0.601. (D) P4-N4 Amplitude; Click, t=0.272, p=0.787; 6kHz, t=0.448, p=0.657; 12kHz, t=0.146, p=0.884; 18kHz, t=0.285, p=0.777; 24kHz, t=0.626, p=0.535; 30kHz, t=0.364, p=0.718. (E) P1 Latency; Click, t=0.209, p=0.836; 6kHz, t=2.016, p=0.051; 12kHz, t=1.034, p=0.308; 18kHz, t=-0.149, p=0.882; 24kHz, t=-0.712, p=0.481; 30kHz, t=1.3156, p=0.198. (F)P1-P2 Latency; Click, t=-1.274, p=0.211; 6kHz, t=-0.574, p=0.569; 12kHz, t=0.421, p=0.676; 18kHz, t=0.197, p=0.845; 24kHz, t=0.201, p=0.841; 30kHz, t=-0.710, p=0.483. (G) P1-P3 Latency; Click, t=-0.186, p=0.854; 6kHz, t=-0.586, p=0.562; 12kHz, t=0.750, p=0.458; 18kHz, t=0.209, p=0.836; 24kHz, t=1.104, p=0.277; 30kHz, t=-0.238, p=0.813. (H) P1-P4 Latency; Click, t=0.203, p=0.841; 6kHz, t=-0.0747, p=0.941; 12kHz, t=1.573, p=0.124; 18kHz, t=1.278, p=0.209; 24kHz, t=-0.502, p=0.619; 30kHz, t=-0.794, p=0.433.

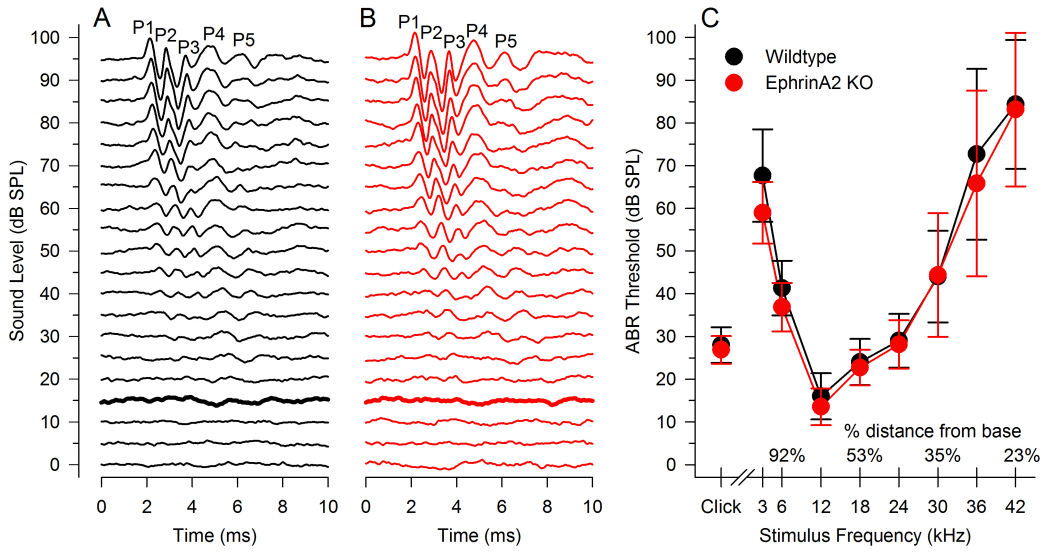

Figure 1

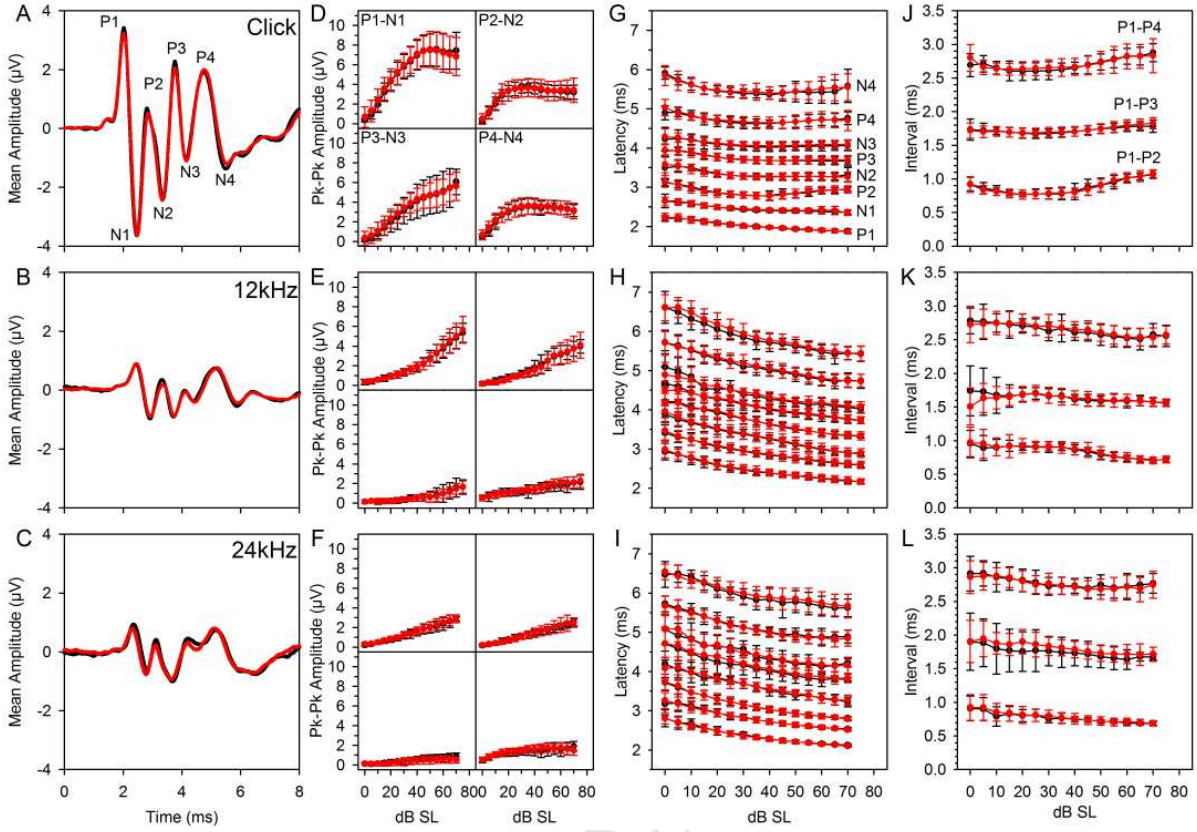

Figure 2

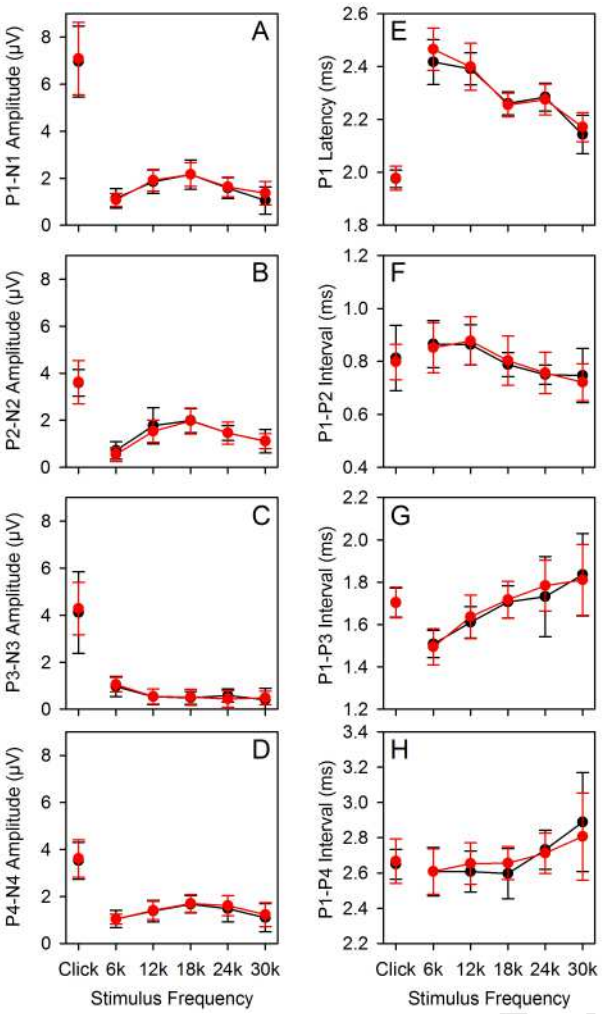

Figure 3

On the role of ephrinA2 in auditory function

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Highlights

- published findings of improved hearing in ephrinA2 knockout could not be replicated
- ephrinA2 knockout mice had comparable auditory sensitivity to littermate controls
- evoked potential wave amplitudes were normal in ephrinA2 knockout mice
- wave latencies and interpeak intervals were normal in ephrinA2 knockout mice